JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 2010, p. 952–956 Vol. 48, No. 3
0095-1137/10/$12.00 doi:10.1128/JCM.01860-09

Effect of Carbon Dioxide on Broth Microdilution Susceptibility Testing of *Brucella* spp.[▽]

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Received 21 September 2009/Returned for modification 2 November 2009/Accepted 11 December 2009

Since some strains of *Brucella* species may require carbon dioxide for growth, a multilaboratory study was conducted to compare broth microdilution susceptibility results using ambient air (AA) and 5% CO_2 incubation conditions. Six antimicrobial agents were tested against 39 *Brucella* isolates. Aminoglycoside MICs tended to be 1 log_2 dilution higher in CO_2 than in AA; tetracycline-class MICs to be 1 log_2 dilution lower in CO_2.

Routine susceptibility testing of *Brucella* spp. is not recommended since the susceptibility pattern of wild-type *Brucella* spp. is fairly predictable, the isolates are fastidious, and the organisms are a potential cause of laboratory-acquired infection (3, 12, 17). In addition, *Brucella* spp. are intracellular pathogens, and like other intracellular pathogens, *in vitro* susceptibility may not always correlate with clinical outcome (1, 3, 28). Typically, brucellosis is treated with dual-antimicrobial therapy to lower the possibility of relapse. The most common combinations are streptomycin (or gentamicin) and doxycycline and doxycycline combined with rifampin (4, 12, 21, 28). Trimethoprim-sulfamethoxazole is recommended as alternative therapy (28), but use of fluoroquinolone therapy is controversial (12, 18, 25). Although there has been little or no resistance reported to routinely prescribed antimicrobials for brucellosis, relapse is still common (3, 5, 22, 25), and development of laboratory-confirmed rifampin resistance has been reported (11).

Brucella suis, *Brucella melitensis*, and *Brucella abortus* are considered potential agents of bioterrorism (6, 24). As with other potential bacterial agents of bioterrorism, engineered antimicrobial resistance is a concern. Antimicrobial susceptibility testing of *Brucella* spp. to identify effective therapeutic and prophylactic agents would be an important response effort in a bioterrorism event. Although no antibiotic regimen has been precisely studied for prophylaxis of brucellosis in humans, combining doxycycline and rifampin or using trimethoprim-sulfamethoxazole alone (for children and pregnant women) has been used successfully in preventing laboratory-acquired disease, although side effects can occur (20, 23, 27).

Many different methods of antimicrobial susceptibility testing, using a variety of media and incubation conditions, have been described for testing *Brucella* spp. (1, 3–5, 12–14, 16, 21, 22, 25, 26). Jevitt et al. (15) developed a standardized method for susceptibility testing of *Brucella* spp. using brucella broth, a method that was adopted by the Clinical and Laboratory Standards Institute (CLSI) in 2006 (9). This initial CLSI method for *Brucella* spp. described a broth microdilution (BMD) procedure using incubation at 35 ± 2°C for 48 h in ambient air (AA). The present study describes a multicenter examination to evaluate incubation in ambient air supplemented with 5% CO_2. Incubation in CO_2-supplemented air is particularly important for some strains of *Brucella* spp. that are especially fastidious, such as *B. abortus* (19). Since many laboratories may not have a dedicated CO_2 incubator in a biosafety level 3 (BSL3) space, incubation using a CO_2-generating system, such as BBL GasPak CO_2 (BD, Sparks, MD) and the BBL Gaspak CO_2 pouch capnophilic system (BD), was evaluated.

Thirty-nine strains of *Brucella* spp. from the Centers for Disease Control and Prevention (CDC) collection were used for this study: 20 *B. melitensis*, 11 *B. suis*, and 8 *B. abortus.* Antimicrobial susceptibility testing was performed in 4 laboratories at 3 institutions. Two laboratories tested both ambient air and CO_2 incubation conditions, and two laboratories performed testing using only one of the incubation conditions. BMD panels were prepared at the CDC with brucella broth (BBL, Sparks, MD) at pH 7 to 7.2 and were shipped to participating laboratories, where they were stored at −70°C until ready for use; all panels were from the same preparation lot number. Antimicrobial powders were obtained from Sigma (St. Louis, MO). The antimicrobial agents and ranges tested were as follows: doxycycline, 0.015 to 8 μg/ml; gentamicin, 0.015 to 8 μg/ml; rifampin, 0.12 to 8 μg/ml; streptomycin, 0.12 to 64 μg/ml; tetracycline, 0.015 to 8 μg/ml; and trimethoprim-sulfamethoxazole, 0.015/0.285 to 8/152 μg/ml. The MIC incubation temperature was 35°C for both atmospheres. CO_2 incubation was accomplished with a single-use gas-generating system which produces an atmosphere that contains 2.5 to 10% CO_2 (BBL product insert). The BBL GasPak CO_2 system was used in 2 laboratories (sites A and B), and the BBL GasPak CO_2 pouch capnophilic system was used in laboratory test site D. Inocula were prepared by the direct colony suspension method in Mueller-Hinton broth from 24- to 48-h cultures grown on 5% sheep blood agar plates (BBL) and incubated in ambient air or in ambient air supplemented with CO_2 by using a gas-generating system if the isolate was dependent upon CO_2 for growth (8). The final volume of brucella broth in the MIC tray

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▽ Published ahead of print on 23 December 2009.

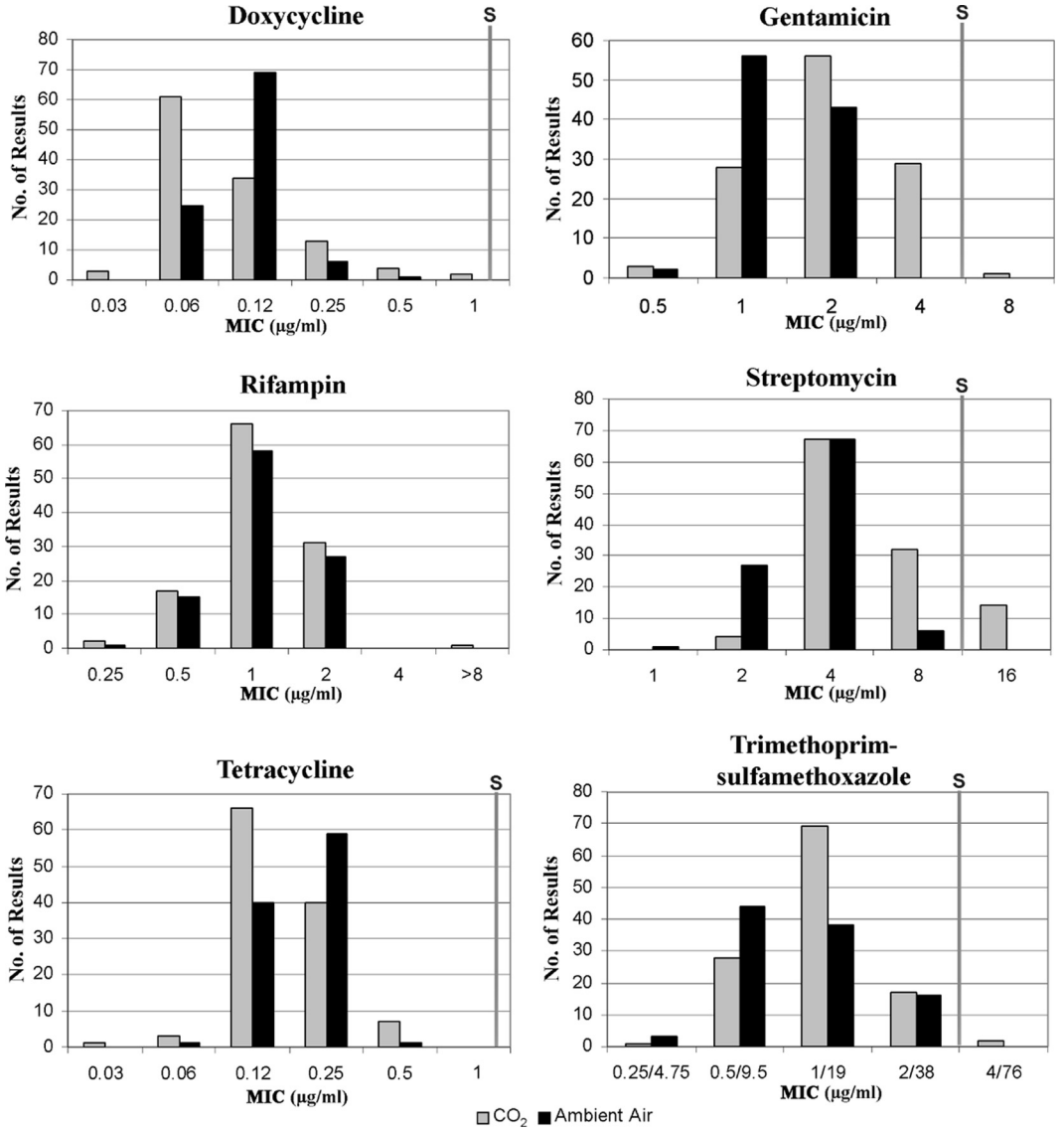

FIG. 1. Bar graphs of MICs under ambient air and CO_2 conditions for six antimicrobial agents tested against 39 *Brucella* isolates at three test sites for each atmosphere. For one site, ambient air data were available for only 31 isolates, while the other two sites each had 35 ambient air results (4/39 isolates required CO_2). S, susceptible. Category divisions are based on the original 2006 CLSI breakpoints; there are no published breakpoints for rifampin (9).

wells was 100 μl per well; 10 μl of diluted inoculum was delivered by a sterile, plastic commercial inoculator system (Dynex, Chantilly, VA). MIC panels were incubated for 48 h, and endpoints were recorded as the lowest concentration of drug demonstrating no macroscopic growth, except for trimethoprim-sulfamethoxazole, where the endpoint was interpreted as the lowest drug concentration inhibiting 80% of the growth when compared to the growth control well.

BMD MIC results from AA incubation and CO_2 incubation for the 39 *Brucella* isolates are shown in Fig. 1. AA MIC results were not available for 4 of the 39 isolates because these isolates would not grow in AA; CO_2 was required for growth in broth and on agar media. The MIC modes for tetracycline and doxycycline were 1 $\log_2$ dilution lower in CO_2 than in AA, while gentamicin and trimethoprim-sulfamethoxazole modes were 1 $\log_2$ dilution higher in CO_2 than in AA (Table 1). The modes for streptomycin MICs in AA and CO_2 were the same (4 μg/ml), but there were 40 more results for which the MIC was 8 or 16 μg/ml in CO_2 than in AA. Fourteen of these 40 streptomycin results were categorized as nonsusceptible (MIC of 16 μg/ml) in CO_2, whereas no results fell into the nonsusceptible category for AA incubation.

Two types of nonparametric statistical methods were used to evaluate differences in MICs from AA incubation versus CO_2 incubation for each of the antimicrobial agents by utilizing Statistical Analysis Software (SAS Institute, Inc., Cary, NC). The first method was the Wilcoxon rank sum test that compares the mean rank MICs for AA versus CO_2 for a given agent. If the mean ranks are not statistically significantly different, the implication is that no significant shift in MIC has

TABLE 1. MIC modes and ranges for six antimicrobial agents tested against 39 *Brucella* isolates at three test sites for each incubation atmosphere[a]

Antimicrobial agent	MIC mode (μg/ml)		MIC range (μg/ml)	
	CO_2	Ambient air	CO_2	Ambient air
Doxycycline	0.06	0.12	0.03–1	0.06–0.5
Gentamicin	2	1	0.5–8	0.5–2
Rifampin	1	1	0.25–>8	0.25–2
Streptomycin	4	4	2–16	1–8
Tetracycline	0.12	0.25	0.03–0.5	0.06–0.5
Trimethoprim-sulfamethoxazole[b]	1	0.5	0.25–4	0.25–2

[a] For one site, ambient air data were available for only 31 isolates, while each of the other 2 sites had 35 ambient air results (4/39 isolates required CO_2).
[b] Only the trimethoprim portion is stated in the table.

TABLE 2. Nonparametric comparison of MICs for six antimicrobial agents tested against 39 *Brucella* isolates at three test sites for each incubation atmosphere

Antimicrobial agent	Mean rank[a]		*P* value[b]
	Ambient air (n = 101)	CO_2 (n = 117)	
Doxycycline	121.0	98.7	0.004
Gentamicin	85.6	130.2	<0.001
Rifampin	108.4	109.6	0.876
Streptomycin	81.6	133.6	<0.001
Tetracycline	119.4	101.0	0.016
Trimethoprim-sulfamethoxazole	98.3	119.2	0.008[c]

[a] n = the number of MIC results for each antimicrobial agent. There were 35 ambient air (4/39 isolates required CO_2) MICs for 2 sites and only 31 at one site, for a total of 101 results. n = 100 available ambient air MICs for doxycycline and rifampin. The mean rank values were calculated by first converting the MICs to whole numbers in a linear fashion (e.g., MICs of 0.25, 0.5, 1, and 2 were converted to 1, 2, 3, and 4, respectively). These relative values were then used for statistical analysis.
[b] Computed using the Wilcoxon rank sum test. A difference is significant if *P* is <0.05. If the mean ranks are not significantly different, the implication is that no significant shift in MIC has been observed based on these sample data. These results are supported by the Kuiper test for all agents except trimethoprim-sulfamethoxazole.
[c] P = 0.110 by Kuiper test.

been observed based on this sample data. If, on the other hand, the mean rank is significantly higher for CO_2 than for AA, this implies a positive MIC shift. The converse implies a positive MIC shift for AA incubation. The second statistical method, the Kuiper empirical distribution function test, was used to compare the empirical distribution of MICs for AA and CO_2. The Kuiper test is used for two-sample data and compares the entire distribution of MICs for AA and CO_2 that is as sensitive in the tails as at the median. Thus, it is possible to observe a significant shift in the mean rank, yet not necessarily to observe a significant shift in the entire distribution of MICs due to less difference in the tails of the distributions.

The results of the nonparametric analysis are shown in Table 2. Doxycycline and tetracycline showed a statistically significant shift to lower MICs under CO_2 conditions, while gentamicin and streptomycin showed a significant shift to higher MICs in CO_2; all were confirmed by the Kuiper test with *P* values of <0.05 (data not shown). Incubation in CO_2 is expected to decrease the pH of the medium, which is known to decrease activity of aminoglycosides and to increase the activity of tetracyclines (2), so higher aminoglycoside MICs and lower tetracycline-class MICs in CO_2 incubation were expected. Twelve streptomycin MICs resulted in a change from susceptible in AA to nonsusceptible when incubated in CO_2. Two additional streptomycin MICs from different CO_2-requiring isolates also had streptomycin MICs in the nonsusceptible range. Similarly, the MIC for one gentamicin result changed from susceptible in AA to nonsusceptible when incubated in CO_2. As a result of these studies, CLSI made two new notations in the 2007 M100-S17 document for susceptibility testing of *Brucella* species (10). The first notation was an additional breakpoint for streptomycin if susceptibility testing is performed in CO_2 incubation; the second notation warned that incubation of broth in CO_2 may increase the MIC of aminoglycosides and decrease the MIC of tetracyclines, usually by 1 doubling dilution (10). Since tetracycline and doxycycline MICs in CO_2 did not exceed 1 μg/ml, 2 $\log_2$ dilutions below the susceptible breakpoint of ≤4 μg/ml, CLSI deemed it unnecessary to provide alternate breakpoints for these drugs if CO_2 incubation was used.

The interlaboratory MIC variability for the four drugs with different results in CO_2 was examined (Table 3). Tetracycline and doxycycline did not show any obvious interlaboratory variation under either AA or CO_2 incubation conditions. For gentamicin and streptomycin, test sites A and D tended to have higher MICs in CO_2 than test site B but little or no variation between sites for AA incubation. This difference could not be explained by the CO_2-generating system since test sites A and B used the same system and site D used another system. It is possible that these differences are the result of inoculum preparation or reader variability.

Trimethoprim-sulfamethoxazole demonstrated a shift to higher MICs in CO_2 using the Wilcoxon rank sum test, but the Kuiper test did not confirm this, giving a *P* value of 0.110. Two trimethoprim-sulfamethoxazole MICs were in the nonsusceptible range when incubated in CO_2: one from a CO_2-requiring strain and one from a strain that did not require CO_2 for growth. All trimethoprim-sulfamethoxazole MICs were in the susceptible range when incubated in AA. Changes in pH are not known to affect trimethoprim but can have a variable effect on sulfonamides (2); therefore, trimethoprim-sulfamethoxazole MICs may be affected by CO_2. The interlaboratory variability of these results was examined (Table 3). Test site C tended to have slightly higher MICs in AA than the other two AA test sites, while test site D had higher MICs in CO_2 than the other two sites for CO_2 incubation. Since the endpoint of this drug is read at 80% inhibition, which is a subjective determination, reader variability is likely for trimethoprim-sulfamethoxazole MICs. Variability in endpoint determination may explain why the two statistical tests did not agree regarding a CO_2 effect on trimethoprim-sulfamethoxazole MICs.

For quality control, MIC panels were tested with *Staphylococcus aureus* ATCC 29213, *Streptococcus pneumoniae* ATCC 49619, and *Escherichia coli* ATCC 25922 in AA and CO_2 atmospheres, as applicable on each day of testing. MICs were read at 24 h and 48 h; all results were within acceptable ranges for *Brucella* susceptibility testing (7, 9), except for one rifampin MIC of 0.25 μg/ml at 24 h and 48 h in CO_2 and one gentamicin MIC of >8 μg/ml at 48 h in AA. There appears to be no CO_2 effect on quality control results, but this is difficult to assess with so few values (Table 4).

TABLE 3. Comparison by laboratory test site of MICs for six antimicrobial agents incubated in ambient air and CO_2 atmospheric conditions for 39 *Brucella* isolates[a]

Antimicrobial agent	Incubation condition	Test site	No. of occurrences at indicated MIC (μg/ml)									
			0.03	0.06	0.12	0.25	0.5	1	2	4	8	16
Doxycycline	AA	A		3	26	2						
		B		13	20	2						
		C		9	23	2						
	CO_2	A		26	11	2						
		B		18	13	4	3	1				
		D	3	17	10	7	1	1				
Gentamicin	AA	A					1	16	14			
		B						21	14			
		C					1	19	15			
	CO_2	A					1	2	19	16	1	
		B					1	23	15			
		D					1	3	22	13		
Rifampin	AA	A				1	3	19	7			
		B					3	21	11			
		C				1	9	16	9			
	CO_2	A					6	32	1			
		B				1	5	19	14			
		D				1	6	15	16			1[b]
Streptomycin	AA	A						1	12	18		
		B							9	24	2	
		C							6	25	4	
	CO_2	A								14	15	10
		B							3	35	1	
		D							1	18	16	4
Tetracycline	AA	A			8	22	1					
		B		1	24	10						
		C			8	27						
	CO_2	A			28	11						
		B			21	13	5					
		D	1	3	17	16	2					
Trimethoprim-sulfamethoxazole[c]	AA	A				3	24	4				
		B					18	17				
		C					2	17	16			
	CO_2	A					9	27	3			
		B				1	15	18	5			
		D					4	24	9	2		

[a] For site A, ambient air (AA) data were available for only 31 isolates (30 for rifampin), while each of the other 2 AA testing sites had 35 AA results (4/39 isolates required CO_2); site C had 34 AA results available for doxycycline.
[b] One rifampin MIC was ≥16 μg/ml.
[c] Only the trimethoprim portion of the 1/19 drug ratio is displayed for the MIC.

In summary, CO_2 increased the aminoglycoside MICs for some *Brucella* isolates by 1 $\log_2$ dilution and lowered tetracycline and doxycycline MICs by 1 $\log_2$ dilution, but only affected the category interpretation of streptomycin. Rifampin MICs were not influenced by CO_2 incubation. For trimethoprim-sulfamethoxazole, CO_2 could not be conclusively proven to affect the MICs for the organisms tested. For MIC testing of *Brucella* spp. in CO_2, additional comments and breakpoints have been approved by the CLSI and published in M100-S17 based upon the results from these investigations (10). A separate breakpoint (≤16 μg/ml susceptible instead of ≤8 μg/ml in ambient air) was given for interpreting streptomycin MICs when CO_2 is used for BMD incubation and warning comments were given for gentamicin, tetracycline, and doxycycline MIC

TABLE 4. Comparison of MICs incubated in ambient air and CO_2 atmospheric conditions for three quality control isolates at 48 h of incubation

Antimicrobial agent	ATCC quality control isolate no.[a]	MIC (μg/ml) range[b]		Acceptable AA MIC range (μg/ml)
		AA	CO_2	
Doxycycline	25922	1–2	1–2	1–4
	29213	0.25–0.5	0.25–0.5	0.12–0.5
	49619	0.12	0.06–0.12	0.03–0.25
Gentamicin	25922	4–≥8[c]	4	1–8
	29213	NA[d]	NA	NA
	49619	NA	NA	NA
Rifampin	25922	8–>8	8	4–16
	29213	NA	NA	NA
	49619	≤0.12	≤0.12–0.25[e]	0.008–0.06
Streptomycin	25922	16–32	16	4–32
	29213	8–32	16–32	8–64
	49619	32	32–64	16–128
Tetracycline	25922	2	4	0.5–4
	29213	0.5–1[f]	0.5	0.25–1
	49619	0.25	0.12–1	0.06–0.5
Trimethoprim-sulfamethoxazole[g]	25922	NA	NA	NA
	29213	NA	NA	NA
	49619	0.5–1	1–2	0.5–2

[a] The quality control isolates represent the following organisms: ATCC 25922, *Escherichia coli*; ATCC 29213, *Staphylococcus aureus*; and ATCC 49619, *Streptococcus pneumoniae*.

[b] There were eight MICs in AA and four MICs under the CO_2 conditions for each drug.

[c] There was one quality control MIC of >8 μg/ml, there were six at 4 μg/ml, and there was one at 8 μg/ml.

[d] NA, not applicable: there are no published breakpoints in brucella broth for this organism and drug.

[e] The lowest attainable MIC for the rifampin dilution series was ≤0.12 μg/ml; one result was 0.25 μg/ml.

[f] There were seven quality control MICs of 1 μg/ml.

[g] Only the trimethoprim portion is displayed.

results for CO_2 incubation. The use of CO_2 should be used only for MIC testing of *Brucella* spp. when it is required for adequate growth, as it can affect the MIC results for aminoglycosides and tetracycline-class drugs.

(This study was presented in part at the 46th Interscience Conference on Antimicrobial Agents and Chemotherapy, San Francisco, CA, 27 to 30 September 2006.)

We thank the staff at CDC's Bacterial Zoonoses Branch and the Division of Food-Borne, Bacterial and Mycotic Diseases (National Center for Zoonotic, Vector-Borne, and Enteric Diseases) for providing many of the *Brucella* spp. used in this study. We thank Brandon Kitchel on the Antimicrobial Resistance Team, Division of Healthcare Quality Promotion at CDC, for making the figure graphics.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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